[Chem. Pharm. Bull. 11 (11) 1456 ~ 1461]

UDC 577.15 : 542.958[546.185]

Morio Ikehara, Eiko Ohtsuka, and Yoshihiro Kodama : Studies on Coenzyme Analogs. XVII.*1 A New Phosphorylating Agent, 2,6-Lupetidylphosphorodichloridate, and Some Investigations on the Selective Phosphorylation.

(*Faculty of Pharmaceutical Sciences, School of Medicine, Hokkaido University**2)

One of the difficulties encountered in the synthesis of nucleoside 5′-phosphates is the appropriate protection of hydroxyl groups. It is rather easier in the case of naturally occurring nucleosides. They have mostly ribose as sugar moiety and could be protected on 2′- and 3′-hydroxyl groups simultaneously by the acetal or ketal formation. While in the case of sugar having no vicinal *cis*-glycol system, it requires at least 3 steps to obtain proper starting material for phosphorylation.[1,2] That is, tritylation*3 of primary hydroxyl groups, acetylation of secondary hydroxyls and removal of trityl group. Especially in the case of purine nucleoside, tritylation often suffered a restriction from the base moiety situated in the neighbor of 5′-hydroxyl group and also acidic treatment for detritylation lowered the yield of desired product.[3] Thus, it seems to be very important to search an reagent phosphorylating exclusively the primary hydroxyl group without any protection on the secondary groups. In this report some observations for such purposes will be described.

Comparing the nature of trityl chloride with other acylating agent, the direct attachment of bulky phenyl groups to the central C atom seems to exert a inhibitory effect to the nucleophilic attack of secondary OH groups. The same configuration around P atom of phosphorochloridate type reagent was not obtained by the replacement of alkyl or aryl group of ester linkage to the bulky one. The freely rotatable P-O-C bond allowed the access of secondary hydroxyl groups. Instead, in order to have a satisfactory steric hindrance around P atom, we must have bulky imidate group around central P atom. With this in mind, we have undertaken the synthesis of 2,6-lupetidylphosphorodichloridate (I). In this compound the bulkier lupetidyl group[4] expected to inhibit the approach of secondary hydroxyl group to phosphorus atom and lupetidyl group will be removed by the mild acidic treatment after phosphorylation as demonstrated[5] earlier.

The synthesis of 2,6-lupetidylphorodichloridate was achieved by the condensation of phosphorus oxychloride with 1.5 equivalents of *cis*-2,6-lupetidine in benzene solution. Fractional distillation of the reaction mixture gave compound (I) as white crystal, b.p_3 81 ~86°, m.p. 70~ 80° in a yield around 14%. Relative low yield could be attributed to the bis-type compound and decompostion during isolation procedure.

The structure of the compound (I) was confirmed by derivating it to the crystalline bis(*p*-nitrophenyl)phosphorolupetidate (II) and lupetidylphosphorodianilidate (III). Deriva-

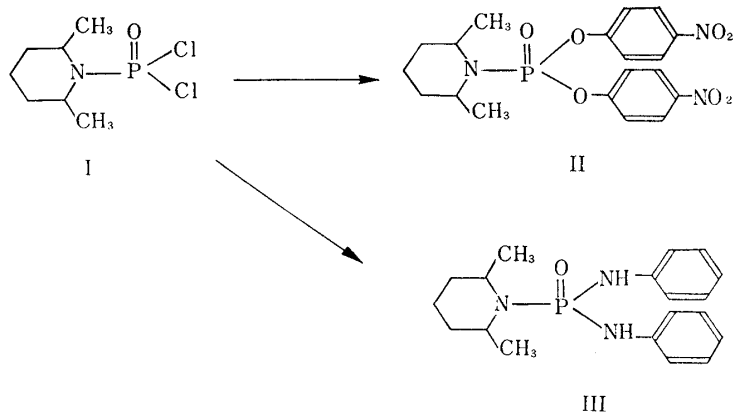
*1 Part XVII. M. Ikehara, H. Tada : This Bulletin, **11**, 1102 (1963).

*2 Kita 12-jo, Nishi 5-chome, Sapporo, Hokkaido (池原森男, 大塚栄子, 児玉喜博).

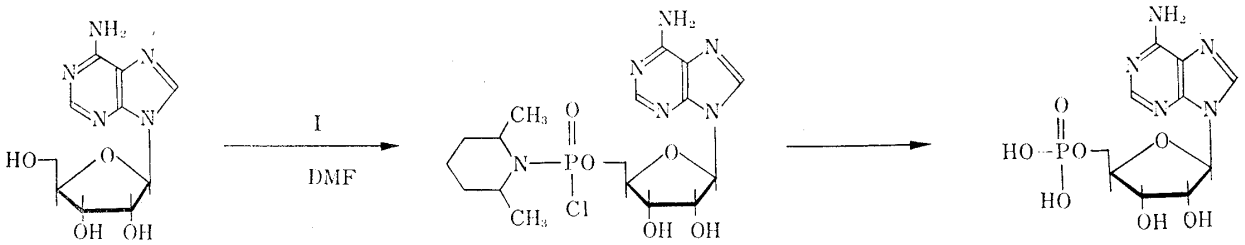
*3 Abbreviations : trityl, triphenylmethyl; lupetidine, dimethylpiperidine; DMF, dimethyl formamide; AMP, adenosine monophosphate; ADP, adenosine diphosphate.

1) H. Bredereck : Ber., **73**, 269 (1940).
2) M. Ikehara, E. Ohtsuka : This Bulletin, **11**, 961 (1963).
3) 2′,3′-Di-O-acetyl derivative of ribofuranoside, *e.g.* adenosine or 2,6-bis-methylmercapto-9-β-D-ribofuranosylpurine[6] was obtained in 20~30% of isolated yield.[7]
4) J. Hine : "Physical Organic Chemistry" p. 78, McGraw-Hill., New York (1956).
5) M. Ikehara, E Ohtsuka : This Bulletin, **11**, 435 (1963).

tive (II) had m.p. 152~154° and from the optical behaviors and elementary analytical data its structure was well defined. Anilidate (III), m.p. 206~208°, was also confirmed from the analytical data as having a correct structure.



I II III



The hydrolysis of reagent (I) in acidic and alkaline media was then investigated. In 0.5*N* hydrochloric acid-ethanol, after reflux for 30 minutes 2/3 of the reagent already decomposed. After 2 hours only inorganic phosphate was detected on paper chromatogram. During the course of hydrolysis a spot corresponding to lupetidyl phosphate had appeared. To our great surprise, in alkaline condition the reagent was hydrolyzed very rapidly. Even at the "0 time" period of hydrolysis with 0.5*N* sodium hydroxide in ethanol, a half of the reagent converted already to inorganic phosphate. After 1 hour's reflux only inorganic phosphate has been detected. In contrast to these results, the acidic hydrolysis of bis(*p*-nitrophenyl)phosphorolupetidate (II) met with difficulty even under a drastic condition. From the evidences obtained above, the access of proton to the fully protected phosphorus atom seems to be completely inhibited. Moreover, it is noteworthy that the lupetidyl group is much labile in alkaline condition than in acidic media in contrast to the ordinary amidate group.[6]

The phosphorylation of adenosine with this reagent in various conditions were investigated next. On the phosphorylation of bared nucleoside, several investigators have reported[7~9] by using excess phosphorylating agent. In these instances, however, main product was 2′,5′- or 3′,5′-diphosphate and the condition was not suitable for our purpose. In the present experiment the ratio of nucleoside to reagent was limited to 1:1 and 1:2. The reaction was carried out by an almost similar procedure to that used in morpholinophosphorodichloridate,[5] except for the solvent. Although in the other experiment[10]

6) J. G. Moffatt, H. G. Khorana : J. Am. Chem. Soc., **83**, 649 (1961).
7) P. A. J. Gorin, L. Hough, J. K. N. Jones : J. Chem. Soc., **1955**, 585.
8) F. Cramer, G. W. Kenner, N. A. Hughes, A. R. Todd : *Ibid.*, **1957**, 3297.
9) J. Baddiley : *Ibid.*, **1958**, 1000.
10) M. Ikehara, E. Ohtsuka : This Bulletin, **11**, 1353 (1963).

dioxane was proved to be useful also in the phosphorylation of bared nucleoside, it was unsuitable for this reaction, because of the low solubility of adenosine. The use of DMF was advantageous for the high solubilizing power and the formation of Vilsmeier complex[11] as postulated by Cramer.[12] Anhydrous pyridine was used also in this experiment as in case of dibenzylphosphorochloridate.[13] The results of the experiments were summarized in the Table I.

Table I. Phosphorylation of Adenosine with Lupetidylphosphorodichloridate

Exp. No.	Ratio (Ad:Rgt.)	Solvent	React. temp. (°C)	Time (min.)	AMP (%) 5′	2′	3′	Higher P
1	1 : 1	dioxane	75	240		(unreacted)		
2	1 : 1	DMF	75	150	15.0	3.0	5.1	+
3	1 : 2	〃	60	150	14.0	2.8	6.0	+
4	1 : 1.5	DMF+dioxane	64	240	12.5	3.8	4.7	—
5	1 : 2	pyridine	60	240	3.5	0.9	1.3	—
6	1 : 2	〃	80	180	5.7	1.8	2.5	+
7	1 : 1 (anhydride)	DMF	30	2840	4.3	1.1	2.1	—

As judged from this data, the most suitable condition for 5′-monophosphorylation of adenosine appeared in the experiment No. 2, in which reaction temperature was maintained at 75°. By this procedure adenosine 5′-monophosphate was obtained in the yield around 15% and the ratio of 5′-, 2′- and 3′-monophosphate was 15:3:5.1.[14] The reagent (I) seemed to be inactive at room temperature, despite of the formation of Vilsmeier complex with DMF. Moreover, anhydride with diphenylphosphate as in the case of P^1-diphenyl P^2-morpholino pyrophosphorochloridate[15] assumed not to be formed when the reagent and diphenylphosphate were combined in DMF. The reaction extent was analyzed by paper chromatography, paper electrophoresis and ion-exchanger chromatography.[16] A typical pattern of the column chromatography was shown in Fig. 1. The separated nucleotides were tested for their uniformity by the paper chromatography with the authentic samples.

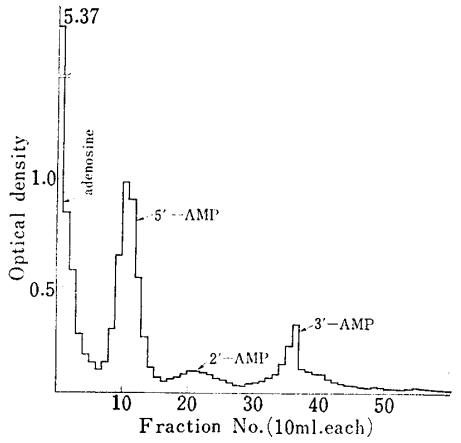


Fig. 1. Pattern of Ion-exchanger Chromatography of Phosphorylation of Adenosine with Lupetidylphosphorodichloridate

Dowex 1 X 8 (formate form)
18 × 0.7 cm, 200～400 mesh
Eluting buffer : 0.125*M* formic acid

11) A. Vilsmeier, E. Haack : Ber., **60B**, 119 (1927).
12) F. Cramer, M. Winter : Chem. Ber., **94**, 989 (1961).
13) J. Baddiley, A. R. Todd : J. Chem. Soc., **1947**, 648.
14) In the separate experiment with P^1-diphenyl P^2-morpholinopyrophosphorochloridate[12] as phosphorylating agent the ratio obtained was 3.5:1.9:4.6. (Experiment by S. Ohtsuka).
15) M. Ikehara, E. Ohtsuka : This Bulletin, **10**, 997 (1962).
16) W. E. Cohn : "The Nucleic Acid" (E. Chargaff, J. N. Davidson, editors) Vol. I, P. 229 (1955).

Although specific phosphorylation of 5'-hydroxyl group was achieved in the range around 15%, this is comparable to the overall yield in the case of phosphorylation of adenosine *via* 2',3'-di-O-acetyl derivative. This yield was also in the same range of the phosphorylation with nucleoside transphosphorylase from carrot[17] which is used in our laboratory for the synthesis of various nucleoside 5'-monophosphates. However, the handlings in a large scale preparation will not be suitable for enzymatic procedure. Moreover, strict specificity of the enzyme prevent the application to the analogs of special structure. Thus, 2,6-lupetidylphosphorodichloridate proved to be useful in the synthesis of 5'-monophosphate of various nucleoside analogs. Further search for more specific and powerful reagent is beng studied.

Experimental

Paper Electrophoresis——0.05*M* triethylammonium bicarbonate, pH 7.5, 20 v./cm., 1 hr. Toyo Filter Paper No. 51A was used.

Paper Chromatography——Solvent A, PrOH-NH_3-H_2O=55:10:35; B, iso-PrOH-NH_3-H_2O=7:1:3; C, saturated $(NH_4)_2SO_4$-H_2O-iso-PrOH=79:19:2. All chromatographies were carried out by ascending technique on Toyo Filter Paper No. 51 A.

2,6-Lupetidylphosphorodichloridate——Into a dry benzene (100 ml.) solution of phosphorus oxychloride (18.5 g., 0.12 mole) 2,6-lupetidine (20.5 g.) was added dropwise under effective stirring, during which the temperature was maintained beneath 30° by the cooling with ice-H_2O. After the addition was completed temperature was raised to 30~40°. Three hours' reaction gave a brown-red solution. Lupetidine hydrochloride (yield, 8.7 g., 65%) was removed by rapid filtration and filtrate and washings (dry benzene) were combined. Benzene and unreacted $POCl_3$ was distilled off *in vacuo* and the residue was applied to the fractional distillation. Distillate ($b.p_3$ 86°) separated after cooling to the white crystal, m.p. 76~81° (3 g., 14.4%), and yellow liquid (3 g.). The crystal was separated by filtration and recrystallized from EtOH, m.p. 84~86°. *Anal.* Calcd. for $C_7H_{14}ONPCl_2$: C, 36.54; H, 6.13; N, 6.08; P, 13.45. Found: C, 36.85; H, 6.39; N, 6.04; P, 13.45. IR: ν_{max}^{Nujol} 1290 cm^{-1} (P=O). Paper chromatography: Rf 0.88 (solvent A), 0.69 (solvent B), accompanied with thin spot having Rf 0.95 (A) and 0.79, 0.69 (B). These were assumed to be partially hydrolyzed product during chromatography. Although the nature of yellow liquid was not investigated precisely, it seems to be a complex mixture of phosphorus-containing compound. The crude reagent obtained by the filtration was used also for phosphorylation and no marked difference between purified crystal was observed.

Acidic Hydrolysis of Lupetidylphosphorodichloridate——Lupetidylphosphorodichloridate (10 mg.) was dissolved in 1 ml. of EtOH, added with 1 ml. of *N* HCl and the whole was kept for standing for 1.5 hr. At each intervals (30, 60, 90, 120 min.) after the beginning of reflux an aliquot was extracted and applied to paper chromatography. Spot was detected by molibdate spray[18] and estimated by visual observation. After 30 min. hydrolysis, ca. 1/3 of the reagent remained as a spot having Rf 0.47 (A), 0.29 (B) and after 2 hr. totally hydrolyzed to inorganic phosphate (Rf 0.24 (A), 0.10 (B)). The spot of Rf 0.47 (A) was assumed to be corresponding to lupetidylphosphate.

Alkaline Hydrolysis of Lupetidylphosphorodichloridate——Lupetidylphosphorodichloridate (10 mg.) was dissolved in 1 ml. of EtOH, added with 1 ml. of *N* NaOH and refluxed. Aliquots were applied to paper chromatograpy (solvent A and B) at 0, 15, 30 and 60 min. after the beginning of reflux. At the 0 min. period, a half of the reagent was decomposed and after 1 hr. merely the spot of inorganic phosphate was detected.

Bis(*p*-nitrophenyl)phosphorolupetidate——Lupetidylphosphorodichloridate (10 mg.) and Na *p*-nitrophenolate (50 mg., dried at 120° for 5 hr. over P_2O_5 at 3 mm. Hg) was refluxed in anhyd. benzene (10 ml.) for 2.5 hr. NaCl was removed by filtration, filtrate was evaporated *in vacuo* and the residue was triturated with Et_2O to afford white amorphous solid. Recrystallization from benzene-petr. ether gave white crystal, m.p. 152~154°. *Anal.* Calcd. for $C_{19}H_{22}O_7N_3P$: C, 52.41; H, 5.09; N, 9.65; P, 7.11. Found: C, 52.41; H, 5.22; N, 9.88; P, 6.97. UV: λ_{max}^{EtOH} 270, λ_{min}^{EtOH} 233 mμ. IR ν_{max}^{Nujol} cm^{-1}: 1350 (NO_2), 1300 (P=O), 1215 (P-O-C), 750 (phenyl).

Acidic Hydrolysis of Bis(*p*-nitrophenyl)phosphorolupetidate——i) Bis(*p*-nitrophenyl)phosphorolupetidate (20 mg.) was dissolved in a mixture of EtOH (5 ml.) and *N* HCl (5 ml.) and heated at 100° for 30 min. Solvent was removed under reduced pressure until the solution became slightly turbid. Storing overnight in a refrigerator gave white needles, m.p. 155~159°. Mixed melting point test and paper

17) M. Tunis, E. Chargaff: Biochim. Biophys. Acta, **37**, 257, 267 (1960).
18) C. S. Hanes, F. A. Isherwood: Nature, **164**, 1107 (1949).

chromatography (solvent A, Rf 0.96; bis(*p*-nitrophenyl)phosphate, 0.92) showed total recovery of starting material.

ii) Heating in 2*N* HCl as 100° for 1 hr. also gave starting material.

Alkaline Hydrolysis of Bis(*p*-nitrophenyl)phosphorolupetidate——Bis(*p*-nitrophenyl)phosphorolupetidate (5 mg.) was dissolved in a mixture of EtOH (1 ml.) and *N* LiOH (1 ml.) and heated at 100° in a fused tube. Aliquot was examined by the paper electrophoresis at 30 and 60 min. period. Two spots corresponding to *p*-nitrophenol (migrating distance 12 cm., R_{AMP} 0.96) and disubstituted phosphate (migrating distance 10.8 cm., R_{AMP} 0.86) were detected.

Lupetidylphosphorodianilidate——Lupetidylphosphorodichloridate (100 mg.) was dissolved in 20 ml. of anhyd. benzene and after the addition of aniline (500 mg.) the whole was refluxed for 30 min. Aniline hydrochloride was removed by filtration and filtrate was evaporated *in vacuo*. Sirupy residue was triturated with Et_2O-petr. ether mixture to afford white amorphous solid. Recrystallization from Et_2O-petr. ether gave crystal, m.p. 206~208°. *Anal.* Calcd. for $C_{19}H_{26}ON_3P$: N, 12.23; P, 9.02. Found : N, 12.03; P, 9.13. UV : λ_{max}^{EtOH} 275.5 mμ. IR ν_{max}^{Nujol} cm^{-1} : 3240 (NH), 1290 (P=O), 745, 692 (phenyl).

Phosphorylation of Adenosine (General procedure)——Adenosine (110 mg., 0.4 mmole) and 2,6-lutidine (43 mg., 0.4 mmole) was dissolved in DMF (6 ml.) by a slight warming. After cooling lupetidylphosphorodichloridate (184 mg., 0.8 mmole) was added. The reaction mixture was heated at 60° with magnetic stirring under exclusion of moisture in a polyethylene bath. 5 minutes after the beginning of the reaction a white precipitate appeared. 1 hr. of the reaction gave a pale yellow color and the increase of the precipitate ceased. Reaction was stopped at 2.5 hr. period and aliquot was examined by paper chromatography. Two spots having Rf 0.24, R_{AMP} 1.1 and Rf 0.57, R_{AMP} 1.0 (solvent A) were revealed by UV irradiation and molibdate spray. In solvent C 2 spots having Rf 0.44 and 0.24 were detected. The former corresponded to adenosine and the latter corresponded to the mixture of 2′-, 3′- and 5′-adenosine monophosphates. H_2O (50 ml.) was added into the reaction mixture (pH became 2.0) and heated at 100° for 30 min. Mixture was evaporated to a small bulk (ca. 2 ml.) and 1/5 of the solution was applied to a column of Dowex 1-X8 (formate, 200~400 mesh, 0.7×18 cm.). Column was eluted with 0.125*M* formic acid. Elution pattern was shown in Fig. 1. The yield of 5′-, 2′- and 3′-AMP calculated from the optical density unit was 15, 3.2 and 5.1%, respectively. Each fractions corresponding to 5′-, 2′- and 3′-AMP was evaporated *in vacuo* and tested for their uniformity and identity with the authentic samples. Paper chromatography (solvent A) showed adenosine, 0.64; 5′-AMP, 0.24; 2′- and 3′-AMP, 0.27. Examinations of the fractions by paper electrophoresis showed the spots corresponding to adenosine, adenosine phosphorolupetidate, AMP, ADP (linear and 2′(3′),5′-) and inorganic phosphate. Migrating distance and R_{AMP} was summarized in Table II. Higher phosphates were not

Table II. Electrophoretic Behavior of Different Compound used in the Experiment

	Mig. distance (cm.)	R_{AMP}	UV	Molibdate	$I0^{-}{}_4$[19]
Adenosine	0.6	0.07	+	−	+
Ad 5′-phosphorolupetidate	3.4	0.45	+	+	+
2′- or 3′-AMP	8.5	1.02	+	+	−
5′-AMP	8.5	1.02	+	+	+
2′(3′),5′-ADP	12.2	1.47	+	+	−
5′-ADP	11.0	1.33	+	+	+
Pi	14.5	1.75	−	+	−

investigated precisely. The reaction carried out in dioxane-DMF (1:1, v/v) mixture or anhyd. pyridine were achieved by analogous procedure as described above. Results appeared in the Table.

Phosphorylation of Adenosine using Diphenylphosphate as Anhydride forming Agent——Lupetidylphosphorodichloridate (45 mg., 0.2 mmole), diphenylphosphate (50 mg., 0.2 mmole) and 2,6-lutidine (43 mg., 0.4 mmole) was dissolved in DMF (1 ml.) and kept in standing for 15 min. at room temperature. The whole was added into a solution of adenosine (54 mg., 0.2 mmole) dissolved in DMF (1 ml.) by a slight heating. The reaction mixture was incubated in a thermostat (31~32°) for 48 hr. After further 12 hr. at room temperature, a white precipitate appeared. Solvent was removed at 50~60° *in vacuo*, dried by codistillation with benzene and 50 ml. of H_2O was added. The solution was heated at 100° for 30 min. Analysis by paper chromatography, electrophoresis and ion-exchanger chromatography as described in general procedure showed the occurrence of 4.3% of 5′-AMP, 1.1% of 2′-AMP, and 2.2% of 3′-AMP. This result seems to indicate that lupetidylphosphorodichloridate did not form the anhydride with diphenylphosphate under the condition investigated.

19) M. Viscontini, O. Hoch, P. Karrer : Helv. Chim. Acta, **38**, 642 (1955).

Authors wish to thank Mrs. T. Tohma and Miss A. Maeda of this Faculty for the elementary analyses. This experiment was financially supported by the Grant-in-Aid for Scientific Research from the Ministry of Education, which was gratefully acknowledged.

Summary

By the reaction of 2,6-lupetidine with phosphorus oxychloride a new phosphorylating agent, 2,6-lupetidylphorodichloridate was synthesized. Unprotected adenosine was phosphorylated with this reagent on 5'-hydroxyl group in a yield around 15%.

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Masuo Akagi, Takayuki Misawa, and Hiroyasu Kaneshima : Studies on the Metabolism of Borate. III.*1 Variations of Fructose 6-Phosphate Levels and Fructose 1,6-Diphosphate Levels in some Organs and Blood after Administration of Borate, and Effects of Boron on Anaerobic Glycolysis.

(*Faculy of Pharmaceutical Sciences, School of Medicine, Hokkaido University,**2 *and Hokkaido Institute of Public Health**3)

During the studies on the biochemical actions of borate, the authors suspected that borate might inhibit the anaerobic glycolysis of glucose. For the purpose of studying the effects of boron upon the sugar metabolism, the authors examined influences of boron upon the levels of fructose 6-phosphate (F6P) and fructose 1,6-diphosphate (FDP) in some organs and blood of the rats and guinea pigs, and also investigated effects of boron upon the anaerobic glycolysis.

It was found that the animals received borate orally in a single dose of 140 mg. or 240 mg./kg. apparently showed a tendency to rise the FDP levels in livers within 4 hours, while they showed no significant change of the F6P levels in livers as well as levels of both F6P and FDP in brains, kidneys and blood. It was also found that the anaerobic glycolysis of glucose in homogenates of livers and brains of the animals was considerably inhibited by borate, and the rate of the inhibition was higher in the former.

Methods and Results

Animals, Diet and Dosage——Rats (0.200~0.250 kg., body wt.) and guinea pigs (0.370~0.500 kg., body wt.) were used. The keeping, diets and the method of administration of borate were the same as the previous report.*4

Determination of F6P and FDP in Biological Materials——F6P and FDP in biological materials were determined spectrophotometrically by the method described by Roe, *et al.*[1]

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*1 Part II. M. Akagi, T. Misawa, H. Kaneshima : Yakugaku Zasshi, **83**, 209 (1963).
*2 Nishi-5-chome, Kita-12-jo, Sapporo (赤木満洲雄).
*3 Nishi-15-chome, Minami-2-jo, Sapporo (三沢隆行, 金島弘恭).
*4 Part I. M. Akagi, T. Misawa, H. Kaneshima : Yakugaku Zasshi, **82**, 934 (1962).
1) J. H. Roe, *et al.* : J. Biol. Chem., **210**, 703 (1954).